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14. ABSTRACT

Chronic myelogenous leukemia (CML) is a progressive disease of the hematopoietic stem cell (HSC). It has long been postulated that BCR-ABL causes genomic instability, which then drives the malignant progression of CML. We propose that BCR-ABL causes a chronic instability through a congruence of events that are accidentally combined to place the genome at risk. In particular, we focus on three events: production of reactive oxygen species (ROS), reduction in the repair of oxidized DNA, and defect in oxidative stress-induced apoptosis. We propose the following three lines of exploratory experiments: 1) Determine the effect of BCR-ABL kinase on the steady-state levels of reactive oxygen species (ROS). 2) Examine the effect of BCR-ABL kinase on the expression and the function of MYH repair pathway. 3) Examine the effect of BCR-ABL kinase on the p53-family of transcription factors. The proposed research may elucidate a pathway for oxidative damage to propel malignant progression in CML and thus identifies specific use of antioxidants to combat CML blast crisis.

15. SUBJECT TERMS
BCR-ABL, tyrosine kinase, reactive oxygen species, oxidative DNA damage, DNA repair, apoptosis

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I. Introduction

Chronic myelogenous leukemia (CML) is a progressive disease of the hematopoietic stem cell (HSC)[1,2]. It has long been postulated that BCR-ABL causes genomic instability, which then drives the malignant progression of CML[1]. This idea is supported by the fact that imatinib-resistant blast crisis results from the re-emergence of BCR-ABL kinase activity rather than progression into BCR-ABL-independent leukemia[3]. Increased mutation rate and chromosomal aberrations have been associated with blast crisis; yet, the underlying causes for these events are unclear. The genetic instability of CML is most likely caused by the BCR-ABL tyrosine kinase. The cytoplasmic BCR-ABL tyrosine kinase activates a host of signal transduction pathways to stimulate cell proliferation. A byproduct of these activities is the production of ROS, which has been detected in cells expressing BCR-ABL[4]. The increased production of ROS can cause several DNA lesions, including strand breaks, oxidative deamination, and the formation of 8-oxo-7,8-dihydroguanine (8oxoG) [5]

We propose that BCR-ABL causes a chronic instability through a conflation of events that are accidentally combined to place the genome at risk. In particular, we focus on three events: production of reactive oxygen species (ROS), reduction in the repair of oxidized DNA, and defect in oxidative stress-induced apoptosis. To test our hypotheses, we propose the following three lines of exploratory experiments: 1) Determine the effect of BCR-ABL kinase on the steady-state levels of reactive oxygen species (ROS). 2) Examine the effect of BCR-ABL kinase on the expression and the function of MYH repair pathway. 3) Examine the effect of BCR-ABL kinase on the p53-family of transcription factors.

The proposed research is highly relevant to the treatment of CML. Recent success in CML therapy, based on the clinical use of imatinib (GLEEVEC) to inhibit the BCR-ABL tyrosine kinase, has been widely acknowledged[3]. While imatinib is effective in suppressing CML during the chronic phase, it is ineffective in combating CML during blast crisis[6,7]. Our idea that reactive oxygen species play a critical role in the malignant transformation of CML, if correct, has important implications for the treatment of CML. Dietary antioxidants have been the subject of interest and controversies in the prevention and treatment of cancer[8]. There has not been a solid scientific basis to explain the sporadic effectiveness of antioxidants in the clinics[9]. The propose research may elucidate a pathway for how oxidative damage can propel malignant progression in CML and thus identifies specific use of antioxidants to combat CML blast crisis.

II. Progress Report (Body)

II-1) Research Objectives

We have proposed to establish MO7e human leukemia cell line expressing a novel FKBP-BCR-ABL protein that is susceptible to regulation by a synthetic chemical dimerizer binding to the FKBP domain. We will then employ established methods to determine cellular levels of reactive oxygen species in response to dimerization and activation of FKBP-BCR-ABL. We will also determine using ELISA assays the abundance of 8oxoG[10,11] in genomic DNA resulting from FKBP-BCR-ABL dimerization. We will use RT-PCR and immunoblotting to detect changes in mRNA and protein levels of Oggl, MTH1 and MYH, three critical players in dealing with 8oxoG, as a result of FKBP-BCR-ABL kinase activity. Immunofluorescence staining will be used to detect alternations in the subcellular localization of Oggl, MTH1 and MYH in response to FKBP-BCR-ABL kinase activity. We will thereafter

determine changes in the rate of 8oxoG clearance from the genome as a result of FKBP-BCR-ABL kinase activity. Changes in the expression and subcellular localization of p53, p63 and p73[12-16] resulting from FKBP-BCR-ABL activation will be evaluated by quantitative RT-PCR, immunoblotting and immunofluoroscence. We will finally determine whether over-production of p53, p63 or p73 restores the apoptotic response of FKBP-BCR-ABL expressing leukemic cells to radiation and chemotherapeutics.

II-2) Progress to Date

Establishment of MO7e cell line for ecotropic retroviral infection to ensure human safety:
MO7e is a human leukemic cell line that has previously been used to study the biological effects of BCR-ABL[17]. Hence, we have chosen it to be the experimental system for this study. To efficiently introduce FKBP-BCR-ABL into this cell line, retroviral-mediated gene transfer is the ideal method. Because MO7e is of human origin, amphotropic retrovirus has always been utilized to transfer genes into these cells. Unfortunately, amphotropic retrovirus poses a threat to human handlers because it is fully capable of infecting humans[18]. Out of concern for safety, we prefer to use ecotropic retrovirus, which only infects rodent cells and does not infect humans. Because BCR-ABL is a known human leukemia gene, we have decided to modify the MO7e cells so that we can use ecotropic virus to transduce the FKBP-BCR-ABL gene and avoid the risk of transducing this gene into humans. Retroviral tropism is determined by species-specific affinity of the viral receptors on recipient cells[19]. We have therefore established a new MO7e cell line that expresses the mouse cell surface receptor for the ecotropic retrovirus.

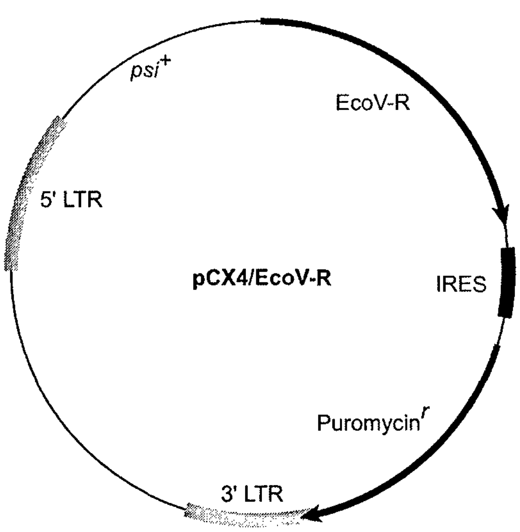

Figure 1. pCX4/EcoV-R retroviral plasmid encoding ecotropic viral receptor (EcoV-R). EcoV-R, ecotropic viral receptor; IRES, internal ribosome entry site; psi+, extended viral packaging signal; LTR, long terminal repeats of retroviruses.

Construction of EcoV-R plasmid Receptors on human cell surface do not interact with the glycoprotein of ecotropic retrovirus. To enable ecotropic infection of human cells, we introduced a receptor for ecotropic retrovirus (EcoV-R) into MO7e cells[20]. An expression cassette of EcoV-R was cloned into an amphotropic retrovirus vector bearing a puromycin-resistance gene (Figure 1). The expressions of both EcoV-R and Puromycin^r are driven from a single promoter in the 5' LTR. An internal ribosome entry sites (IRES)[21,22] is interposed between EcoV-R and Puromycin^r to allow translation of Puromycin^r off the same transcript as EcoV-R. The elements of 5' LTR, 3' LTR and psi$^+$ (extended viral packaging signal) ensures the long transcript of the construct to be packaged in retroviruses when expressed in packaging cell lines.

Generation of amphotropic retrovirus from EcoR-V plasmid The plasmid of pCX4/EcoV-R (Figure 1) was transfected into Phoenix helper-free amphotropic packaging cells[23] to derive

amphotropic viruses encoding EcoV-R. MO7e cells were then infected with the recombinant viruses prior to selection for puromycin resistance over a period of one week. The resultant cell lines were tested for susceptibility to infection with ecotropic retroviruses encoding BCR-ABL. Western blotting for BCR-ABL on protein extracts of the infected cells (not shown) have confirmed our success in establishing a MO7e cell line amenable to infection by ecotropic viruses that are safe to humans.

Construction of ecotropic retrovirus plasmid encoding FKBP-BCR-ABL protein:

Key to the proposed study is an engineered BCR-ABL protein that, unlike its wild type, stays monomeric by itself but oligomerizes into an active state upon the binding of a chemical dimerizer to the FKBP domain strategically placed at its N-terminus (Figure 2)[2]. The FKBP domain is an arrangement of FK506 binding motifs in tandem, which are from the so-called FK560-binding protein (FKBP)[24,25]. The FKBP domain replaces the critical 63 BCR amino acids at the N-terminus of BCR-ABL proteins. As these 63 amino acids constitute the oligomerization domain of BCR-ABL [26], the resultant FKBP-BCR-ABL fusion protein cannot form oligomers in the absence of dimerizer and is predicted to lack the hyper kinase activity associated with BCR-ABL. The chemical dimerizer AP20187 through its interaction with the FKBP enables the oligmerization of FKBP-BCR-ABL to bring about the hyperactivity of BCR-ABL[25,27]. We have successfully engineered the very large FKBP-BCR-ABL fusion construct using the recently developed recombineering technology[28,29] and cloned it in a retrovirus vector (pMSCV).

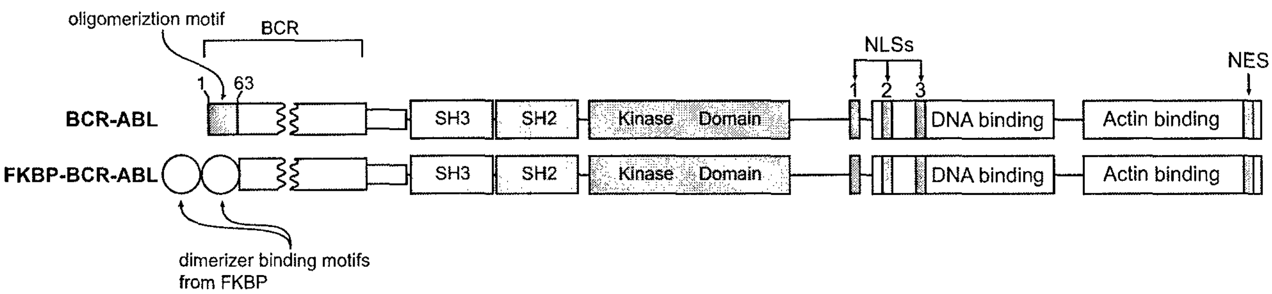

Figure 2. The schematics of FKBP-BCR-ABL in comparison with BCR-ABL. Two copies of dimerizer binding motifs replace the native oligomerization motif at the N-terminus of BCR-ABL. The dimerizer motif is derived from the FK506 binding domain of FK506 binding protein (FKBP) and engineered through mutagenesis to achieve optimal affinity for the synthetic dimerizer AP20187.

Generation of ecotropic retroviruses encoding FKBP-BCR-ABL

The FKBP-BCR-ABL expression cassette was cloned into a ecotropic retroviral vector pMSCV (Invitrogen). The construct was transfected into BOSC23 ecotropic packaging cell line[30], which contains two stably integrated plasmids encoding *gag* and *pol* besides ecotropic *env* genes to package the transcripts of the construct into virions. The culture media of the cells are collected in 4 days of transfection, into which retroviruses are secreted. The collected media are subject to unltra-centrifugation to pellet down the retroviruses. The pellet is then re-suspended and enriched for retrovirus by filtration through a size-exclusion chromatograph column. The resultant retroviruses, now highly concentrated are stored at -80°C for future application[31].

III. Key research accomplishments

- We have established a modified MO7e cell line, MO7e-Eco, that expresses the ecotropic retroviral receptor to enable ecotropic retroviral-mediated gene transfer, thus, reducing the risk of exposing investigators to retrovirus that carries the BCR-ABL human oncogene.

- We have employed a novel recombineering technology to construct the FKBP-BCR-ABL fusion gene in a retroviral vector.

- We have generated ecotropic retrovirus to transduce the FKBP-BCR-ABL gene.

IV. Reportable outcomes

Research activities during the funding period have resulted in the creation of the following new reagents that will faciliate CML research-

- MO7e-Eco cells.

- Recombinant DNA that express a novel FKBP-BCR-ABL fusion protein.

- Packaged ecotropic retrovirus that transduces the FKBP-BCR-ABL gene.

V. Conclusions

Biological conclusions relevant to the proposed hypothesis are not available at this time. With the established cell line and reagents, we are in position to conduct the proposed experiments to test our hypothesis. Biological conclusions will be forthcoming in the next six months of this study.

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VII. Appendices
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